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Lack of Clinical Evidence for Involvement of Hepatitis C Virus Interferon-α Sensitivity-Determining Region Variability in RNA-Dependent Protein Kinase-Mediated Cellular Antiviral Responses

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The hepatitis C virus (HCV) interferon-α (IFN-α) sensitivity-determining region (ISDR) has been shown to suppress double-stranded RNA-dependent protein kinase (PKR) activity in vitro in a yeast PKR expression system. Since variability of ISDR was shown to correlate with nonresponsiveness to IFN-α therapy in chronically HCV-infected patients, it has been suggested that prototype ISDR might be a viral inhibitor of cellular PKR. The present study evaluates the biological significance of ISDR variability in situ, relating it to PKR-mediated cellular antiviral responses within the liver. ISDR variability was determined in patients chronically infected with HCV genotypes 1a, 1b, and 3a by direct sequencing using liver-derived RNA preparations as starting material. As surrogate parameters for PKR-mediated cellular responses, hepatic endogenous IFN-α gene expression as well as *MxA* expression were analysed by a competitive, quantitative reverse transcription-polymerase chain reaction technique. Irrespectively of intra- or intergenotypic ISDR amino acid substitutions, ISDR variability was found not to correlate with endogenous hepatic IFN-α or with hepatic *MxA* gene expression. The data suggest that at least two prominent PKR-mediated cellular responses might be largely unaffected by HCV ISDR variability. ***J. Med. Virol. 61:29–36, 2000.***

KEY WORDS: hepatitis C virus; interferon sensitivity-determining region; nonstructural protein 5A; interferon-α; double-stranded RNA-dependent protein kinase

INTRODUCTION

Cellular double-stranded (ds) RNA-dependent protein kinase (PKR) is expressed constitutively at low levels [Meurs et al., 1990; Thomis et al., 1992]. Its mRNA expression can be increased up to five- to 10-fold by exogenous interferon-α (IFN-α) [Meurs et al., 1990; Thomis et al., 1992]. Enzymatic activity of PKR, however, is induced and regulated on the protein level. Activation of PKR protein requires binding of dsRNA and, subsequently, an autophosphorylation step. The phosphorylated form of PKR is active biologically. It exerts antiviral activities, since it catalyses the phosphorylation of the α subunit of the eukaryotic translation initiation factor 2, resulting in inhibition of both viral and cellular protein synthesis and death of the infected cell [reviewed by Clemens and Elia, 1997] (Fig. 1).

Besides its inducibility by type I interferons, PKR also has been shown to be involved in the induction of type I interferon production, for example, after viral infections [Der and Lau, 1995; Kirchhoff et al., 1995]. This might represent a kind of amplification process: Once IFN-α-induced PKR enzyme has been activated by dsRNA, it might not mediate just direct antiviral responses but also a reinforced IFN type I induction (Fig. 1B). Dependency of type I IFN gene expression on PKR activity is evidenced by loss-of-function phenotypes in stable transformants of the pro-monocytic cell line U937 [Der and Lau, 1995] and NIH3T3 cells [Kirchhoff et al., 1995] and by the use of an inhibitor of protein kinase with proven selectivity for PKR, 2-aminopurine, in various experimental systems with

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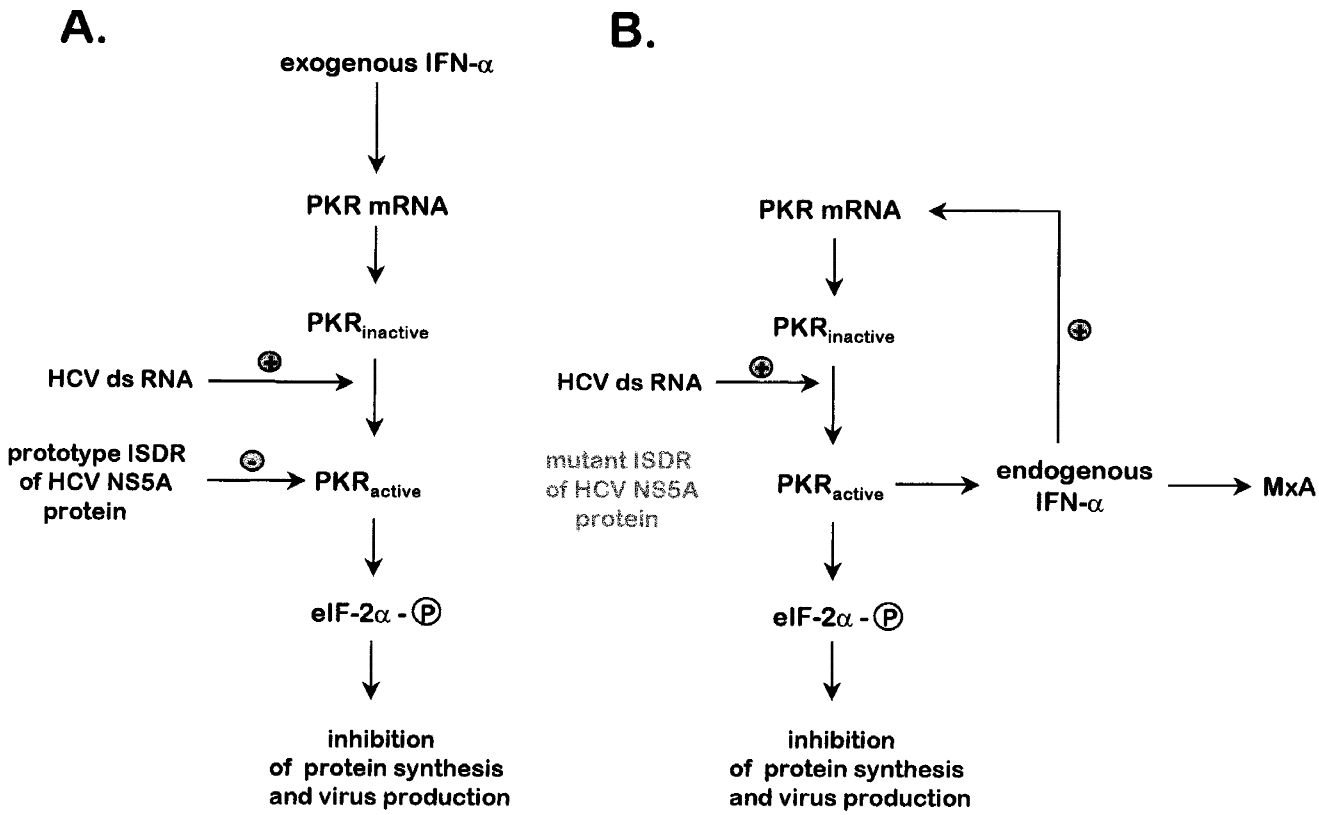

Fig. 1. Putative interaction of double-stranded (ds) hepatitis C virus (HCV) RNA and the prototype interferon-α (IFN-α) sensitivity-determining region (ISDR) of HCV nonstructural protein 5A (NS5A) with RNA-dependent protein kinase (PKR) activation and subsequent activation of endogenous IFN-α genes and *MxA*-specific transcripts. PKR mRNA and PKR enzyme are constitutively expressed at low levels within the cell. The enzyme is biologically inactive, however, unless it is activated by dsRNA, that is, of viral origin. The active form of PKR is able to phosphorylate the α subunit of the eukaryotic translation initiation factor 2 (eIF-2α), resulting in general inhibition of protein synthesis, a possible defence mechanism against viral infections. **A:** It has been shown that the presence of the nonmutant prototype HCV ISDR within NS5 interferes with the activation process, resulting in suppression of enzyme activity [Enomoto et al., 1995, 1996; Chayama et al., 1997; Gale et al.,1997, 1998]. This inhibitory mechanism might be advantageous for HCV, especially under therapeutic conditions—for example, when PKR expression is up-regulated severalfold by the application of exogenous IFN-α. **B:** The absence of prototype HCV ISDR, for example, the presence of mutant forms of ISDR within the infected cell, might allow the active PKR enzyme to exert its functions. Besides its effects on cellular protein synthesis via phosphorylation of eIF-2α, PKR has been shown to be involved in the induction of endogenous IFN-α [Tiwari et al., 1988; Zinn et al., 1988; Wathelet et al., 1989; Der and Lau, 1995; Kirchhoff et al., 1995). Endogenous IFN-α, in turn, might reinforce cellular PKR expression, thus amplifying this early host defence system. Moreover, IFN-α is a well-known and specific activator of the MxA protein [Jakschies et al., 1994; Roers et al., 1994].

divers inducers of interferon [Tiwari et al., 1988; Zinn et al., 1988; Wathelet et al., 1989].

Type I IFNs comprise IFN-α and IFN-β. Whereas human IFN-β is encoded only by a single gene, 14 human IFN-α encoding nonallelic genes plus four pseudogenes are recognised [Fitzgerald-Bocarsly, 1993]. To circumvent technical difficulties resulting from the multispecies nature of interferons, that is, to capture all IFN-α species on the protein or at the mRNA level, interferon expression is analysed either by its biological activity (e.g., by suppressing viral replication in in vitro systems) or by surrogate parameters for IFN-α activity. The most specific surrogate parameter known for IFN-α action is activation of the *MxA* gene [Jakschies et al., 1994; Roers et al., 1994].

Viruses are involved in several molecular activities through which they interfere with PKR activity, providing them with protection against this early host defence system [reviewed by Gale and Katze, 1998]. The nonstructural protein 5A (NS5A) of hepatitis C virus (HCV) has been shown to interact with the catalytic domain of PKR, both in a cell-free in vitro system and in yeast cells co-expressing human PKR and NS5A, resulting in repression of enzyme activity [Gale et al., 1997, 1998] (Fig. 1A). This interaction requires the IFN-α-sensitive phenotype of the putative IFN-α sensitivity-determining region (ISDR), a region within NS5A whose variability has been shown to correlate with IFN-α responsiveness in therapeutic interventions in type 1b-infected Japanese patients [Enomoto et al., 1995, 1996; Chayama et al., 1997].

The biological relevance of HCV ISDR variability for therapeutic IFN-α responsiveness is difficult to prove, however, because of the lack of appropriate animal models and tissue culture systems for HCV infection. Yet to address the question of biological significance of amino acid substitutions within the ISDR, the study relates ISDR variability to two cellular antiviral responses, in which PKR activation is known to be involved, namely IFN-α and *MxA* gene activation.

PATIENTS AND METHODS

Patients

After obtaining informed consent, serum and liver biopsy specimens were collected consecutively from 28 patients infected chronically with HCV (12 women and 16 men, aged 18–67 years; mean age, 44.6 years). HCV infection was diagnosed by the presence of anti-HCV antibodies and HCV RNA in serum. The chronic nature of their illness was established by histopathologic examination according to established criteria described by Mihm et al. [1997]. Patients with concomitant active hepatitis B virus or human immunodeficiency virus infection and those who had ongoing problems with alcohol or drug abuse were excluded. The study was approved by the local ethics committee of the Georg-August-University, Göttingen.

Determination of Hepatitis C Virus Genotypes

Determination of HCV genotypes was carried out according to the method of Okamoto et al. [1992,1993], with subsequent restriction enzyme analysis as described previously [Mihm et al., 1996].

Determination of Nucleotide and Deduced Amino Acid Sequences Within Nonstructural Protein $5A_{2209\text{-}2248/2215\text{-}2254}$

RNA was isolated from either 200 μl serum using a commercially available extraction kit (RNA-Clean system; AGS, Germany) or liver biopsy specimens, as described by Mihm et al. [1996]. One-fifth of the serum RNA preparation or 100 ng of the liver tissue RNA preparation was subjected to reverse transcription. Reactions were carried out at 42°C for 30 min in a total volume of 20 μl containing 10 mmol/L HEPES at pH 6.9, 0.2 mmol/L ethylenediaminetetraacetate at pH 8.0, 50 mmol/L Tris-Cl at pH 7.5, 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiotreitol, 0.5 mmol/L deoxynucleoside triphosphate, 20 U RNase inhibitor (RNA-guard; Pharmacia, Germany), 15 U Superscript II-RT (GIBCO BRL, Germany), and 50 ng of primers specific for genotype 1b (Geno1b/2 5′-TCTTTCTCCGT-GGAG-GTGGTATTGG-3′), 1a (1a antisense 5′-GAGACTTCC-GCA GGATTTCT-3′), or 3a (Geno3/3 5′-GTCCGGTCT-AGCCCAGATAG-3′), respectively. The reaction was stopped by heat inactivation. One-fourth of the cDNA was amplified by polymerase chain reaction (PCR), carrying out 30 cycles with denaturation for 30 sec at 94°C, primer annealing for 40 sec at 60°C, and extension for 60 sec at 72°C. Amplification was followed by a final extension step for 7 min at 72°C.

Each reaction contained 10 mmol/L Tris-Cl at pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.01% gelatine, 200 μmol/L dNTP, 0.5 U *Taq* polymerase (Boehringer Mannheim, Germany), and 30 ng of each primer of the primer pairs specific for genotype 1b (Geno1b/1 5′-TGGAT-GGAGTGCGGTTGCACAGGTA-3′, Geno1b/2), 1a (1a sense 5′-TGACGTCCAT-GCTCACTGAT-3′, 1a antisense), or 3a (Geno3/1 5′ -CHGTGCTG ACCTC-GATGTTG-3′, Geno3/3), respectively, in a total volume of 50 μl. Starting with 1 μl of the first-round product, a second amplification reaction was performed using 125 ng of the internal primer pairs Geno1b/3 (5′-CAGGTACGCTCCGGCGTGCA-3′) and Geno1b/4 (5′-G GGGCCTTGGTAGGTGGCAA-3′), 1a nested sense (5′ -CCTCCCATATAACAGCAGAG-3′) and 1a nested antisense (5′-CGAAGGAGTCCAGAATCACC-3′), and Geno3/2 (5′-GCG CGCGGGTCCCCTCCATC-3′) and Geno3/3, respectively, for type 1b, 1a, and 3a amplification. One-tenth of the second-round product was analysed by agarose gel electrophoresis.

Amplification products were purified by removing excess nucleotides and oligonucleotides using a commercially available purification kit (QIAquick PCR purification kit; QIAGEN, Germany). Nucleotide sequences were determined for both strands with the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Germany) on an ABI 373A DNA sequencer (Applied Biosystems, Germany). Sequences were analysed using the program package GCG (Wisconsin Sequence Analysis Package, Version 8.1; WI).

Analysis of *MxA* and Interferon-α Transcript Expression by a Competitive, Quantitative Reverse Transcription-Polymerase Chain Reaction Technique

Transcript expression of *MxA* and IFN-α was quantified by a competitive reverse transcription-polymerase chain reaction (RT-PCR) procedure using internal cDNA standards. The amount of specific transcripts was then related both to the amount of albumin and to the amount of β-actin-specific transcripts. The principle and application of the method have been described previously [Mihm et al., 1996]. Furthermore, specific gene expression data are related to the total amount of RNA subjected to RT-PCR. Only gene expression data that are largely independent of the chosen reference transcript are considered.

For quantification of transcripts specific to the human *MxA* gene, an analogous technique has been established using an internal standard constructed to be recognized by and to compete for oligonucleotide primers derived from the human *MxA* mRNA sequence (*MxA* forward 5′-CT-GTGGCCATACTGCGAGGA-3′ nucleotides 7 to 26; *MxA* reverse 5′-ACTCCTGACAGT-GCCT-CCAA-3′ nucleotides 469 to 488) (gene bank accession M30817). Co-amplification of target cDNA corresponding to 32 ng of total cellular RNA and internal standard was carried out in 36 cycles with an initial denaturation step at 94°C for 5 min, annealing at 60°C for 50 sec, extension at 72°C for 50 sec, and a final extension step at 72°C for 10 min. The amplification product of the *MxA*-specific transcript is 482 bp, and that of the internal standard is 320 bp in length. Specificity of the amplification product was confirmed by restriction enzyme analysis. Briefly, digestion by *Ava*I yielded DNA fragments of 446 bp and 36 bp in size, and digestion by *Sty*I yielded products of 379 bp and 103 bp in size, respectively, whereas amplification products

were not digested by *Xho*I. Restriction endonucleases were obtained from Boehringer Mannheim.

For hepatic IFN-α gene expression, the technique was adapted by using a degenerated primer pair able to recognize the individual IFN-α transcript species, as described by Der and Lau [1995]. Co-amplification of target cDNA corresponding to 32 ng of total cellular RNA and internal standard was then carried out in 36 cycles, with an initial denaturation step at 94°C for 4 min, annealing at 68°C for 50 sec, extension at 72°C for 50 sec, and a final extension step at 72°C for 10 min. The amplification products of IFN-α-specific transcripts are 372 bp, and those of the internal standard are 506 bp in length.

Statistical Analysis

Significance levels of the difference between mean values were determined by means of the parametric *t* test for independent samples or ANOVA, respectively, as indicated. With respect to the competitive, quantitative RT-PCR procedure, variability of individual transcript expression data was calculated to be 102% with a standard deviation of 107% (n = 25), ranging between 0% and 320% (interassay variability).

RESULTS

Comparison of Hepatic Interferon-α Sensitivity-determining Region Variability Within Isolates From Patients Infected with HCV Types 1b, 1a, and 3a

Liver biopsy specimens from 28 patients infected by HCV genotypes 1b (n = 17), 1a (n = 6), and 3a (n = 5) were analysed for HCV ISDR nucleic acid variability by a direct sequencing procedure according to the method of Enomoto et al. [1995, 1996]. Deduced ISDR amino acid sequences of type 1b-infected patients resemble prototype sequences (n = 5) as well as intermediate sequences with one amino acid substitution (n = 12) (Fig. 2). Related to prototype isolate HCV-J, ISDR sequences from type 3a or 1a isolates, respectively, might exert genotype-specific amino acid exchanges in addition to intrinsic substitutions (Fig. 2). With regard to type 1a isolates, databank sequences differ from the prototype HCV-J sequence in three positions. Deduced amino acid exchanges observed within the isolates investigated comprise those that are distinct from types 1a and 1b sequences and those that are distinct from type 1a sequences but which “restore” the prototype 1b sequence (Fig. 2). Considering these latter exchanges, the type 1a isolates under investigation differ in two positions from prototype 1b at maximum. With respect to type 3a isolates, databank sequences differ in eight to nine positions from HCV-J. Within the samples analysed, no other non-synonymous nucleic acid substitution could be detected. Type 3a isolates from the patients under investigation thus differ from the prototype HCV-J sequence in eight to nine positions in total (Fig. 2).

Resemblance of Hepatic Interferon-α Sensitivity-Determining Region Variability to Serum Interferon-α Sensitivity-Determining Region Variability

As a control, direct sequencing of ISDR was undertaken using total RNA from liver biopsy specimens as well as RNA preparations from corresponding serum samples. Material from six patients was analysed. Although some differences in the ISDR nucleotide sequence could be observed in five of six patients, none of these nucleotide exchanges was found to resemble a non-synonymous one, compared with the liver-derived sequence (Fig. 2).

Failure of Hepatic Interferon-α Sensitivity-Determining Region Variability to Be Related to Hepatic Endogenous Interferon-α and to Hepatic *MxA* Transcript Expression

Liver samples were analysed by a quantitative, competitive RT-PCR procedure for endogenous IFN-α gene expression and for *MxA* transcript expression. For quantifying endogenous IFN-α expression, a degenerated primer pair was used that was capable of recognising the total array of IFN-α transcript species [Der and Lau, 1995]. ISDR variability failed to correlate with endogenous IFN-α transcript expression irrespective of whether type 1b-infected patients with wild-type and intermediate-type ISDR were compared with each other (Fig. 3A) or whether type 1b-infected patients were compared with non-type 1b-infected patients (Fig. 3A). Analysing both inter- and intragenotypic ISDR amino acid substitutions in patients infected chronically with HCV genotypes 1a, 1b, and 3a, ISDR variability did not correlate with hepatic expression of *MxA*-specific transcripts (Fig. 3B).

DISCUSSION

HCV ISDR variability has been shown to be related to IFN-α responsiveness in Japanese patients infected by genotype 1b [Enomoto et al., 1995, 1996; Chayama et al., 1997]. Two European studies could not confirm those findings [Khorsi et al., 1997; Squadrito et al., 1997; Odeberg et al., 1998]. European groups, including ours, are unable to prove this relationship because of an insufficient number of cases of type 1b-infected patients responding to therapy in a sustained fashion [Hofgärtner et al., 1997; Zeuzem et al., 1997; our own unpublished observations]. Patients studied within these groups, however, are infected mostly by HCV harbouring nonmutant ISDR. The latter studies therefore do not necessarily contradict the concept that ISDR variability confers IFN-α sensitivity; they might explain geographical differences in IFN-α responsiveness as well [Herion and Hoofnagle, 1997].

Provided that the HCV-J prototype virus NS5A protein is indeed an inhibitory modulator of the human PKR enzyme and that amino acid substitutions interfere with its function, as suggested by the experiments of Gale and colleagues [1997, 1998], any amino acid

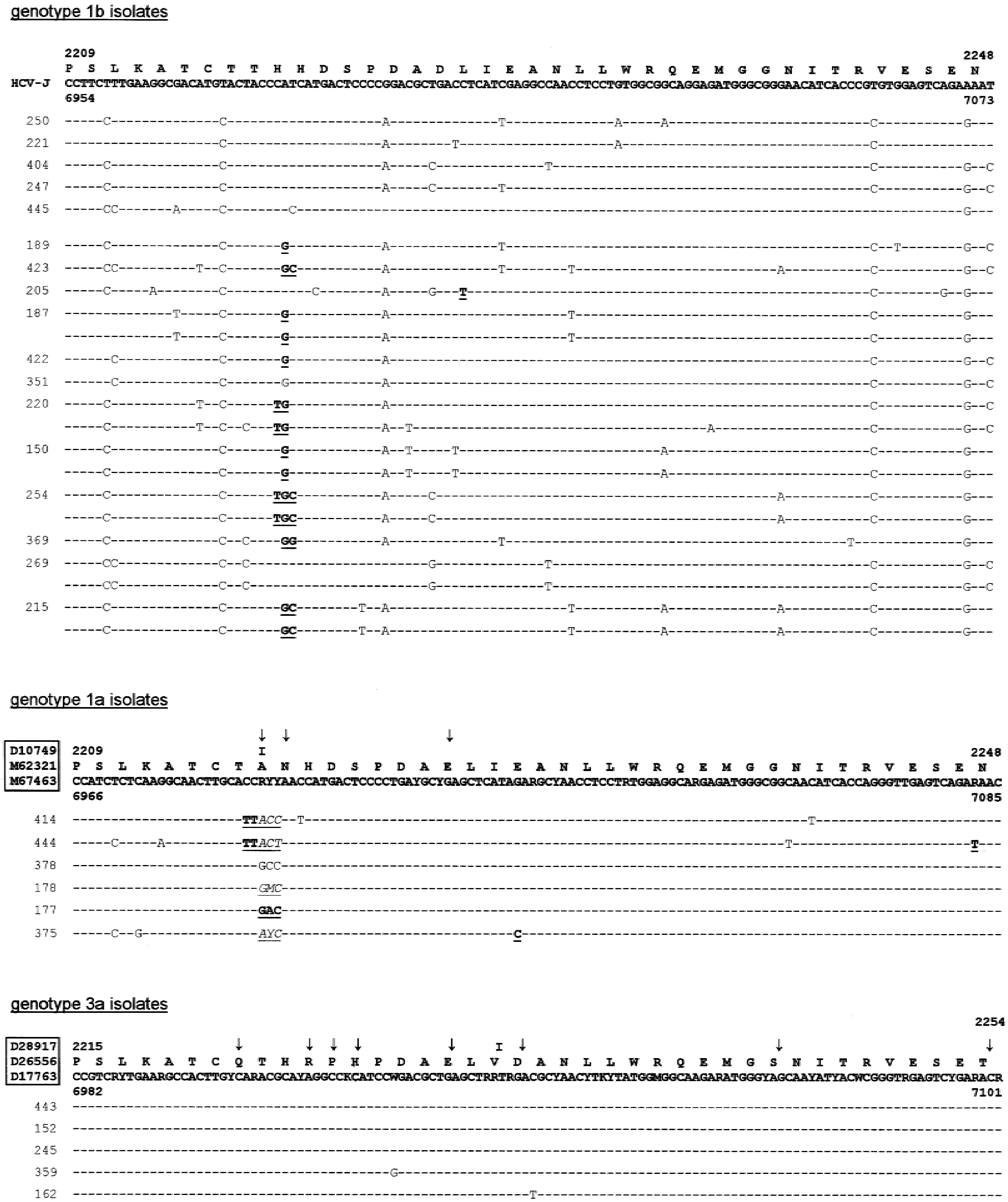

Fig. 2. Nucleic acid variability within the interferon-α sensitivity-determining region (ISDR) of hepatic hepatitis C virus (HCV) isolates. Nucleotide sequences of the ISDR were determined by means of a direct sequencing procedure from preparations of total RNA obtained from liver biopsy material of patients infected by HCV genotypes 1a (n = 6), 1b (n = 17), and 3a (n = 5). With respect to genotype 1b, sequences were compared with the prototype sequence of the isolate HCV-J. Dotted lines indicate identity with the prototype sequence; nucleotide substitutions are given. Non-synonymous substitutions are indicated by bold, underlined characters. Type 1b-infected patients showed prototype ISDR (n = 5) as well as intermediate-type ISDR sequences (n = 12). Types 1a and 3a sequences, respectively, were compared with databank sequences, as indicated. The deduced amino acid sequence of type 1a databank sequences differs from the HCV-J sequence in three positions and that of type 3a databank sequences in eight to nine positions, as indicated by arrows. Intragenotypic exchanges are indicated by underlined bold characters. Amino acid substitutions within type 1a isolates, resulting in restoration of the prototype HCV-J sequence, are indicated by italic underlined characters. In addition to liver tissue, corresponding serum samples were analysed for ISDR variability from six type 1b-infected patients. Serum sequences are given below the respective hepatic sequences without further indication.

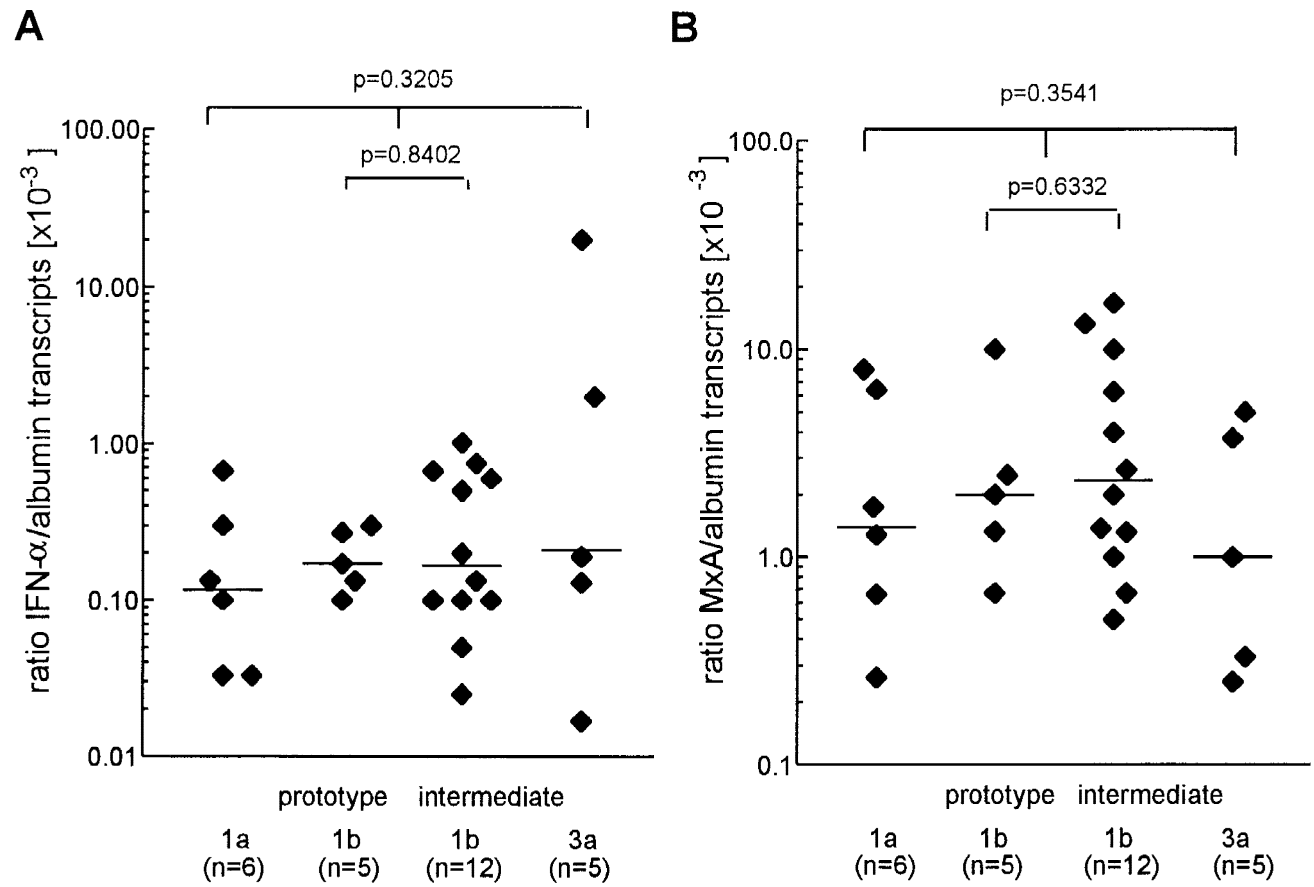

Fig. 3. Lack of a correlation of both inter- and intragenotypic interferon-α (IFN-α) sensitivity-determining region (ISDR) variability and hepatic IFN-α and *MxA* gene expression. RNA preparations from liver biopsy specimens obtained from patients chronically infected with hepatitis C virus (HCV) types 1a, 1b, and 3a were analysed for IFN-α-specific transcript expression **(A)** and for *MxA*-specific mRNA expression **(B)** by a quantitative, competitive reverse transcription-polymerase chain reaction procedure, as described in Patients and Methods. According to ISDR variability, type 1b-infected patients were further divided into those with prototype and those with intermediate-type ISDR sequences. No statistically significant differences could be detected when endogenous IFN-α or *MxA* gene expression were compared among patients infected with types 1a, 1b, or 3a (*P* values by ANOVA). Moreover, no significant differences were detected when patients with prototype and intermediate-type 1b ISDR sequences were compared with each other (*P* values by *t* test for independent samples). Comparable nonsignificant results were obtained when data are related to β-actin as a reference transcript (A: P = 0.3234 by ANOVA, for comparison of genotypes 1a, 1b, and 3a; P = 0.9644 by t test for independent samples, for the comparison of prototype and intermediate-type 1b isolates. B: P = 0.1389 by ANOVA, for comparison of genotypes 1a, 1b, and 3a; P = 0.9285 by t test for independent samples, for the comparison of prototype and intermediate type 1b isolates). Patients with HCV genotype 1a, 1b, or 3a infections were not found to differ significantly with respect to total albumin gene expression (P = 0.1440, Kruskal-Wallis).

substitution should interfere with activity irrespective of whether substitutions are due to intra- or intergenotype variability. In fact, the available data on ISDR variability of non-1b subtypes and IFN-α responsiveness are completely in line with this assumption [Kurosaki et al., 1997; Zeuzem et al., 1997; Sáiz et al., 1998]. Among types 2a- and 2b-infected patients, Kurosaki and colleagues found a rate of response comparable to type 1b-infected patients with mutant types of ISDR [Sáiz et al., 1998]. Databank sequences from HCV types 2a and 2b differ from prototype genotype 1b in a deletion of four amino acids and more than 14 amino acid substitutions within the ISDR [Sáiz et al., 1998]. This finding is comparable to those in type 1a-infected patients [Zeuzem et al., 1997] and type 3a-infected patients [Gale et al., 1998]. Taking the isolate HCV-J as a reference for genotype 1a isolates, subtype-specific amino acid sequences differ in no more than three positions, reflecting a nonmutant-type ISDR. Nine of ten type 1a-infected patients studied by Zeuzem and colleagues [1997] show three or fewer substitutions; no one showed a response to the drug. Related to the genotype 1b prototype, the ISDR of HCV type 3a varies in a genotype-specific way in eight or nine amino acid positions, and these groups of patients show a responsiveness to IFN-α treatment comparable to HCV type 1b-infected patients with a mutant-type ISDR [Sáiz et al., 1998].

Direct proof of the biological significance of ISDR variability for cellular PKR activity, as suggested by the in vitro experiments undertaken by Gale and colleagues [1997, 1998] in a yeast cell system, cannot be construed, because of the lack of appropriate animal models and in vitro systems [Gale and Katze, 1998]. We thus chose to compare ISDR variability with PKR-mediated cellular responses within liver biopsy mate-

rial obtained from chronically HCV-infected patients. Surely, direct measurements of PKR enzymatic activity—for example, by determining the phosphorylation status of the eukaryotic translation initiation factor 2α—would be a more direct approach and the technique of choice. The available number of single liver biopsy specimens sufficient for a large number of quantitative RT-PCR estimates established in our lab, however, sets the limit to the techniques on the protein level. The absence of correlations of hepatic ISDR variability to both hepatic IFN-α transcript expression and hepatic *MxA* expression does not necessarily argue against an interaction of ISDR and PKR. The activity of PKR might be the result of an interplay of viral regulators as well as of cellular modulators, for example, $P58^{IPK}$ or $P52^{rIPK}$ [Gale et al., 1998], including as yet unknown components. The data also do not refute other than PKR-mediated, ISDR-directed antiviral processes. For instance, a recent report by Fukuma et al. [1998] focused on the role of ISDR variability for the transcriptional activity of the NS5A protein. The data presented, however, dispute the biological significance of ISDR variability for at least two PKR-mediated cellular antiviral responses in a clinically relevant in vivo intrahepatic situation.

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